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Genetic Diversity and Streptomycin Sensitivity in *Xanthomonas axonopodis* pv. *punicae* Causing Oily Spot Disease in Pomegranates

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Abstract: *Xanthomonas axonopodis* pv. *punicae* (Xap) causes bacterial blight disease in pomegranates, often leading to 60–80% economic loss. In absence of a suitable Xap-resistant variety, the near-monoculture of the susceptible variety, Bhagwa, has aggravated the problem further. In recent times, Xap has spread to different geographical regions, indicating the wide adaptability of the pathogen. Moreover, lower sensitivity of Xap towards streptocycline containing streptomycin sulphate and tetracycline sulphate (9:1) under field conditions is frequently reported. Therefore, the current study was undertaken to assess the genetic variability of Xap isolates using SSR markers, their in vitro sensitivity towards streptomycin was evaluated, and the probable molecular basis of acquired resistance was studied. Two highly diverse isolates showed extreme differences in their pathogenicity, indicating the highly evolving nature of the pathogen. Moreover, all the isolates showed less than 50% growth inhibition on media containing 1500 μg/mL streptomycin, indicating a lower level of antibiotic sensitivity. On the molecular level, 90% of the isolates showed the presence of *strA-strB* genes involved in streptomycin metabolism. Additionally, G to A transitions were observed in the *rpsL* gene in some of the isolates. The molecular data suggest that horizontal gene transfer (*strAB*) and/or spontaneous gene mutation (in *rpsL*) could be responsible for the observed lower sensitivity of Xap towards streptomycin.

Keywords: bacterial blight; *Xanthomonas axonopodis* pv. *punicae*; pomegranate; antibiotic resistance genes; streptomycin

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1. Introduction

The pomegranate (*Punica granatum* L.) is an economically important fruit crop globally, and India is the global leader in pomegranate cultivation (0.283 million ha) as well as production (3.186 million MT) [1]. Over the last two decades, greater consumer awareness towards its innumerable health benefits has increased its market demand, resulting in alluring monetary returns from this horticultural crop, especially in India. Among popular pomegranate varieties grown in India, Bhagwa has become a highly preferred commercial variety due to its demand in export as well as local markets, and hence, it has occupied more than 86% of the area under pomegranate production in India [2]. The popularity of this variety can be witnessed through the remarkable increase in pomegranate area (108.93%), production (268.53%), and export value (455.68%) in comparison to 2003–04 (DAC, 2020). However, this variety is highly susceptible to *Xanthomonas axonopodis* pv. *punicae* (Xap), causing oily leaf spot or bacterial blight disease. Therefore, its production and processing are severely affected by blight when environmental conditions are conducive for the causative organism to grow and cause the disease. The peak period of blight incidence

coincides with the Mrig bahar (i.e., the flowering on pomegranates occurs during the rainy season: June–July), and the estimated loss due to blight during this bahar is around 60 to 80% [3]. Moreover, no resistant cultivars of pomegranate against Xap are currently available; therefore, the farmers growing Bhagwa often resort to indiscriminate use of chemicals to control or prevent the onset of the bacterial disease [3].

In India, streptocycline, which contains streptomycin sulphate and tetracycline hydrochloride (9:1), is a recommended control measure for several fruits and vegetables, including pomegranates. It is sprayed on plants at a concentration of 0.5 g/L in combination with other bactericides such as bronopol [4]. However, under the integrated disease and pest management schedule (IDIPM), it is recommended only as an emergency control measure for blight on pomegranates [4,5], but the farmers often use this antibiotic indiscriminately for preventing bacterial blight incidence on pomegranates. The dose is often not regulated and may result in excessive usage and deposits in the soil. Moreover, streptomycin is produced by the soil microbe *Streptomyces griseus* [6], and Xap is also known to remain dormant in infected plant debris lying in the soil for some time until favorable conditions for re-infecting pomegranate hosts are encountered [7,8]. Therefore, the constant exposure of bacteria to antibiotics may lead to the development of antimicrobial resistance [9], as reported in human or animal pathogens [10], as well as plant pathogens [11,12].

The most probable mechanism of developing resistance against streptomycin is through the horizontal transfer of genes encoding the phosphotransferase enzyme, which is used by antibiotic-producing *Streptomyces* for self-protection against the antibiotic [13]. Two such major determinants of resistance are: (i) a gene pair *strA-strB* (*strAB*) and (ii) the *aadA* gene and its variant alleles. *strAB* is associated with transposon Tn5393 [14], while *aadA* is associated with integrons [15]. The other possible mechanism of resistance is the occurrence of mutations in *rrs* or *rpsL* genes, leading to alteration in the antibiotic binding site on ribosomes [16]. Mutational resistance often enables the bacteria to grow even in presence of high concentrations of the antibiotic [17].

Streptomycin resistance in the *Xanthomonas* species infecting tomatoes [11], citrus [18], or rice [19] has been previously reported, but the information on the sensitivity of Xap towards streptomycin is scarce. A recent study [20] reported streptomycin sensitivity in Xap isolates collected from pomegranate orchards in the Indian state of Karnataka. The authors used 10 Xap isolates and reported the presence of *strA-strB*. The isolates showed lower in vitro sensitivity to streptomycin, and the growth inhibition of the isolates on the highest concentration of streptomycin (1500 μg/mL) was 35%, which was suggested to be due to the presence of these genes. However, the study did not report any variability or mutations in the *rpsL* genes harbored by these isolates. Therefore, the current study was designed with the following objectives: (i) to collect Xap isolates from pomegranate orchards covering varied geographical locations in India and test their pathogenicity, (ii) to confirm their identity and analyze genetic diversity using SSRs, (iii) to evaluate their sensitivity to streptomycin in vitro, and (iv) to detect the presence of antibiotic resistance genes (*strA-strB*) and analyze the variability in the *rpsL* genes in these isolates.

2. Material and Methods

2.1. Collection of *Xanthomonas axonopodis* pv. *punicae* (Xap) Isolates

Pomegranate leaves/fruits with oily spots (which are the most prominent symptoms of bacterial blight) were collected from different geographical regions of India during 2015–2018 (Table 1, Figure S1). The single-colony isolates were obtained using the standard protocol followed in the lab. Briefly, the leaf/fruit samples were surface sterilized using 2% sodium hypochlorite (2–3 min), followed by washing with sterile deionized water (thrice) and blot drying. The plant samples were then macerated and streaked onto nutrient glucose agar (NGA) media. The plates were incubated at 28 ± 1 °C for 3–4 days. Colonies that developed after 48 h were picked and identified based on colony color, texture, morphology, and the production of characteristic brown-pigmented fuscan. This brown colored pigment,

observed after 5 days of incubation at 28 °C, is characteristic to Xap and has been used as a phenotypic trait for identification of Xap [21,22]. In total, 22 Xap isolates were used for further studies.

Table 1. Details of geographical origin of Xap isolates.

Sr. No.	Isolate Code	State in India	GPS Coordinates
1.	XAP-61	Maharashtra	18°14′00″ N 75°41′34″ E
2.	XAP-63	Maharashtra	17°50′00″ N 75°51′00″ E
3.	XAP-88	Maharashtra	18°17′21″ N 75°41′15″ E
4.	XAP-99	Maharashtra	17°43′46″N 75°17′19″ E
5.	XAP-104	Maharashtra	17°40′30″ N 75°19′36″ E
6.	XAP-114	Maharashtra	17°30′39″ N 75°27′07″ E
7.	XAP-119	Maharashtra	17°23′52″ N 75°48′03″ E
8.	XAP-134	Maharashtra	17°31′27″ N 76°12′19″ E
9.	XAP-93	Maharashtra	17°29′04″ N 75°14′05″ E
10.	XAP-117	Maharashtra	18°34′48″ N 73°52′25″ E
11.	XAP-96	Maharashtra	18°37′35″ N 75°04′43″ E
12.	XAP-98	Maharashtra	17°43′08″ N 75°50′38″ E
13.	XAP-108	Maharashtra	21°33′18″ N 74°28′12″ E
14.	XAP-83	Maharashtra	17°43′09″ N 76°01′26″ E
15.	XAP-92	Himachal Pradesh	31°57′33″ N 77°06′32″ E
16.	XAP-60	Karnataka	13°08′05″ N 77°29′45″ E
17.	XAP-97	Karnataka	16°10′08″ N 75°39′41″ E
18.	XAP-112	Karnataka	17°19′47″ N 76°50′03″ E
19.	XAP-94	Telangana	16°36′12″ N 77°43′35″ E
20.	XAP-110	Tamil Nadu	13°52′26″ N 75°34′01″ E
21.	XAP-115	Uttar Pradesh	26°31′36″ N 79°49′46″ E
22.	XAP-116	Rajasthan	25°45′07″ N 71°23′48″ E

2.2. Pathogenicity Assay

To check whether all the isolates were pathogenic or not, their pathogenicity was tested on healthy pomegranate plants. Inoculum was prepared by multiplying bacteria in nutrient glucose broth (NGB) at 28 ± 1 °C for 48–72 h with constant shaking at 100 rpm. Bacterial cell suspension with 10^7–10^8 cells mL^{-1} ($OD_{600\ nm}$ = 0.2–0.3) was used to inoculate 120-day-old pomegranate plants of the Bhagwa variety. The plants were covered 24 h prior to inoculation and for 24–48 h after inoculation to conserve moisture and ensure optimum infection [21]. Data for disease incidence and severity were recorded at regular intervals, which were converted to percent blight incidence and percent blight severity [21]. Lesions observed on leaves were classified as small and big lesions based on their size; lesion area was then measured. The isolates were individually isolated from the infected pomegranate leaves to prove Koch postulates.

2.3. Confirmation of Identity of Xap Isolates and Detection of Variability Amongst Isolates Using SSR Markers

The Xap isolates were inoculated in broth and incubated for 48 h at 28 ± 1 °C. The cells were pelleted down and used for genomic DNA (gDNA) isolation by a HiMedia bacterial genomic DNA isolation kit, as per the manufacturer's protocol. The gDNA was used as a template for PCR, using Xap-specific XopQ primers that produce 190 bp amplicon [23]. PCR was performed in a thermocycler (HiMedia, India) with 50 ng of gDNA, 10µM primer forward and reverse each (Table 2), 5 µL of 2 X PCR master mix (HiMedia), and molecular grade sterile water in a final volume of 10 µL. The PCR program was set as following: initial denaturation at 94 °C for 4 min, followed by 35 cycles of denaturation (94 °C for 15 s), annealing (58 °C for 30 s), extension (72 °C for 45 s), and the final extension at 72 °C for 10 min. The PCR product was resolved on 2% agarose gel and visualized under UV.

Table 2. List of primers used in the current study.

Name	Forward (5′-3′)	Reverse (5′-3′)	Amplicon Size (bp)
XopQ	GCGAGGAACTTGGAATGCTC	AGGTCGAAGGCTTTTTGCG	190
strA	CCAAGTCAGAGGGTCCAATC	TGACTGGTTGCCTGTCAGAG	760
StrB	TAGATCGCGTTGCTCCTCTT	ACGTTTCGCAACCTGTTCTC	758
rpsL	CAAGCGACCACCTACAAGAGT	GTACTTGGAACGGCCTTGAC	315

For the SSR assay, eighteen primers were selected and screened on 22 *Xap* isolates to evaluate molecular diversity (Table 3). The PCR reaction was performed with 20 ng of genomic DNA in 10 µL reaction volume following PCR conditions, as mentioned above, with annealing Tm (55 °C for 30 s). The PCR products were separated on 3% metaphor agarose gels containing 0.5 µg/mL EtBr and 1 X (TBE) running buffer at 130 V for 4 h, visualized and gel-documented using the Vilbert dourmet (France) system.

Table 3. Details of 18 XAP-SSR primers used for the genetic diversity analysis.

Sr. No.	Primer	Motif	Primer Sequence F/R (5′-3′)	MAF	Allele No.	Gene Diversity	PIC	Expected Amplicon Size (bp)
1	XAP_SSR42	(CG)7	GACACAGACCAACGGATTC/ GGTGACATTGACCTGCTC	0.70	2	0.42	0.33	253
2	XAP_SSR38	(CGG)6	CACCGGCACACTCAATAC/ ATGCATCCTGCTGCTGAT	0.65	2	0.46	0.35	160
3	XAP_SSR59	(CG)6	GAGCGGTTCACAGGAAAT/ GTCGACGAACAACAGCAT	0.75	2	0.38	0.30	223
4	XAP_SSR60	(CG)7	AGGGTTGAGCGTGTTTCT/ CACTGGATCTGACCATCAAG	0.75	2	0.38	0.30	249
5	Xap_SSR28	(ACC)5	AGCGACCAGTCCATCTATC/ CTCCAGCAGGTAGCTCAG	0.56	2	0.49	0.37	291
6	XAP_SSR6	(TGC)4	CTTCCTCGATCATCACCTC/ CGCAGTGATCTTCGTCAT	0.61	2	0.48	0.36	212
7	XAP_SSR22	(CCA)5	ATGCGAGGTACCATGCTC/ CTCTGCCTCAAGGTCTCTTT	0.78	2	0.35	0.29	278
8	XAP_SSR27	(CT)7	GTTGCTTCCTACGACAGG/ GCGCTTCACAGATCGTAT	0.55	2	0.50	0.37	254
9	XAP_SSR24	(GCA)4	CAGCGATGTGGTGATTTC/ GAGCATGCTGGTCAGAAC	0.60	2	0.48	0.36	228
10	XAP_SSR43	(CAT)4	CTGAACATCGTGGTGCTG/ CTGGCTGTTCTGGATCAC	0.53	2	0.50	0.37	252
11	XAP_SSR33	(CG)6	CCTGATCAGCCGGTATTC/ CACACCACACGGTCCATC	0.71	2	0.42	0.33	300
12	XAP_SSR53	(CCA)4	AGATCCAGACCTGTTCGAC/ CTGAACCATCTGCGTACC	0.53	2	0.50	0.37	247
13	XAP_SSR13	(TGG)4	GTCGATCTGGTGGAAGAAC/ AGGAACGAGGAAAACTGC	0.88	2	0.21	0.19	254
14	XAP_SSR17	(CGC)4	GACATGATCGACGTCTCC/ CAGTTGTCCTTGACCTCGT	0.53	2	0.50	0.37	233
15	XAP_SSR10	(GCG)4	CTGACCATCGTCAATTCC/ CATAGGCGATGTTGTTGG	0.68	2	0.43	0.34	283
16	XAP_SSR35	(AGC)4	GTACAGCTCGGATCAACG/ AGGCTTACCAGATCACCAC	0.80	2	0.32	0.27	227
17	XAP_SSR65	(GCC)4	CTTGGGAAGTAACCACCAG/ CGGTGCTGTCAATAGGTT	0.78	2	0.35	0.29	252
18	XAP_SSR23	(GTC)4	GTCAGTTGCAGTGCGTAAC/ GGCTGATTACCTCGTGACT	1.00	1	0.00	0.00	283
	Mean			0.69	1.94	0.40	0.31	

MAF: major allelic frequency; PIC: polymorphic information content.

2.4. Data Analysis

The PCR amplicons of each SSR marker obtained in all the isolates were scored manually as binary data. The marker data were then analyzed to estimate the following marker parameters: the number of alleles (Na), major allelic frequency (MAF), gene diversity (GD), and polymorphic information content (PIC), using PowerMarker v.3.25 [24]. The data sets were also analyzed using the SIMQUAL option to generate pair-wise Jaccard's similarity coefficient [25] using NTSYS-pc version 2.11 W [26]. The similarity matrices thus generated were utilized for the construction of dendrograms using the UPGMA (unweighted pair group method with arithmetic average) algorithm and SAHN clustering. Furthermore, a principal component analysis (PCA) was carried out using NTSYS-pc version 2.11 W [26] in order to have a deeper understanding of the diversity of the Xap isolates.

2.5. Screening for Streptomycin Sensitivity In Vitro

The in vitro antibiotic sensitivity of the Xap isolates was checked using the filter paper disc method. Briefly, sterile filter paper discs were immersed in solutions of streptomycin with different concentrations (250, 500, 750, 1000, 1250, and 1500 µg/mL) for 30 min. NGA media were inoculated with Xap, and then the discs were placed at the center of the plates. The plates were then incubated at 28 ± 1 °C, and zone of inhibition (ZOI) was recorded after 24 h, as per the below formula [20].

$$Inhibition\ \% = 100 - \left[\left\{\frac{(Diameter\ of\ colony\ in\ the\ contorl\ plate) - Inhibition\ zone\ of\ treatment}{(Diameter\ of\ colony\ in\ the\ control\ plate)}\right\} \times 100\right]$$

The screening experiments were performed in triplicate. The data thus obtained were analyzed statistically using WASP (Web Agri Stats Package) software (https://ccari.icar.gov.in/waspnew.html, accessed on 10 March 2022).

2.6. Molecular Confirmation of Streptomycin Resistance Genes

Since all the isolates were resilient to streptomycin, we investigated the molecular basis of this resistance. To check for the presence of the gene pair *strA-strB* (*strAB*), PCR amplification was carried out using gene-specific primers (Table 2). Briefly, PCR was performed in a thermocycler (HiMedia, India) with 50 ng of gDNA, 10 µM primer each, 5 µL of 2 X PCR master mix (HiMedia), and molecular grade sterile water in a final volume of 10 µL. The PCR program was set as follows: initial denaturation at 94 °C for 3 min, followed by 35 cycles of denaturation (94 °C for 45 s), annealing (58 °C for 45 s), extension (72 °C for 1 min), and the final extension at 72 °C for 10 min.

2.7. Molecular Characterization of rpsL Gene

Next, to check for the occurrence of mutations in the *rpsL* gene, this gene was amplified using the *rpsL* primers (Table 2), and the PCR program was set as mentioned above. The amplicons were sequenced using Sanger sequencing at a commercial facility (Eurofins, Bangalore). The sequences thus obtained were searched against the NCBI database using homology search (nBLAST and xBLAST), and after validation, deposited at GenBank NCBI with accession numbers (OK416031-OK416035, OK483036-OK483041, and OK626267-OK626270). Nucleotide sequences were compared and aligned using BioEdit version 7.0.5.3 [27] (http://www.mbio.ncsu.edu/BioEdit/bioedit.html) and MEGA 4 software (http://www.mega.software.net) [28] and verified by comparing with the neighbor-joining method using maximum likelihood in MEGA 4 software for the bootstrap values (1000 replicates) to draw a phylogenetic tree. To predict the coding sequences, all the sequences successfully matched by BLAST were translated into proteins using ORF finder software on the NCBI website (https://www.ncbi.nlm.nih.gov/orffinder/, accessed on 10 March 2022). The longest translated protein was selected for further analysis through batch CD-NCBI search (https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi, accessed on 10 March 2022). Finally, multi-aligning was finished by the ClustalW Multiple function of

BioEdit version 7.0.5.3 [27] and viewed through the graphic view option to identify amino acid changes in the protein domains in comparison to the reference *rpsL* gene protein.

3. Results

3.1. Identity Confirmation and Genetic Diversity Analysis

In the current study, infected leaf samples were collected from pomegranate orchards from different geographical locations in India. A total of 22 isolates were successfully isolated that showed growth characteristics similar to Xap and resembled it morphologically. All the isolates showed the amplification of a 190 bp PCR fragment (Figure 1), which is specific to the XOPQ effector in Xap [23]. Further, the pathogenicity assay revealed that all the isolates initiated characteristic blight symptoms within 14–20 days post inoculation (dpi) under greenhouse conditions (Table 4). However, differences were observed among various isolates for % disease severity (% DS). It ranged from 10 to 86% amongst the isolates (Figure 2). Twelve isolates showed more than 40% DS. The highest severity (86.7%) was observed in the case of isolate number 117, which was collected from Maharashtra, while the least (10.4%) was observed in the case of Xap-92, which was isolated from Himachal Pradesh. Furthermore, pomegranate plants were challenged inoculated with Xap isolates, and leaves showing symptoms were used for the isolation of the pathogen. All the isolates were re-isolated from the infected leaves, proving Koch's postulates. The leaf lesion area was calculated for each isolate. The small lesion area ranged between 3.5 mm^2 and 7 mm^2, while the area for bigger lesions ranged between 10 mm^2 and 25 mm^2 (data not shown).

Genetic diversity amongst the Xap isolates (n = 20) was analyzed using SSR markers (n = 18) (Table 3). Based on the results, the isolates could be grouped into two major clusters (Figure 3). Cluster I contained 5 isolates, and cluster II contained the remaining 15 isolates. Xap-97 and Xap-116 displayed the maximum value for Jaccard's similarity coefficient (0.80), indicating that these two isolates were highly similar (Figure S2). The disease severity exhibited by the two isolates also did not show much of a difference. On the other hand, Xap-60 and Xap-92 displayed the minimum Jaccard's similarity coefficient (0.13) (Figure S2) and were grouped into two different clusters (Figure 3), indicating a high diversity between them. These two isolates also highly differed in their level of pathogenicity in terms of disease severity, Xap-92 being highly pathogenic (86.7%), while Xap-60 displayed low pathogenicity (27.3%), and can therefore be called as different pathotypes (Table 4).

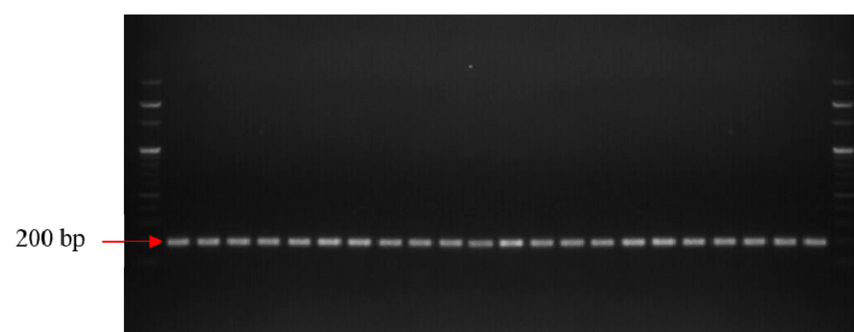

Figure 1. Gel electrophoresis image showing amplification of XOP-Q in all 22 isolates of Xap.

Table 4. Correlation between different parameters of Xap isolates investigated in the current study.

S. No.	Isolate Code	Location	*rpsL* Mutation	*strA/B*	ZOI @ 1500 µg/mL (Non-Transformed)	Antibiotic Resilience/Sensitivity	% DS	Days Taken for Symptom Manifestation	Pathogenicity
Cluster 1									
1.	XAP-60	KA	NA	- +	34.07	S	27.3	3 weeks	low
2.	XAP-112	KA	NA	- +	31.19	S	42.0	2 weeks	high
3.	XAP-93	MH	NA	+ +	34.44	S	27.5	3 weeks	low
4.	XAP-117	MH	NA	+ -	32.96	S	10.4	3 weeks	low
5.	XAP-119	MH	No	+ +	28.52	R	40.7	3 weeks	high
Cluster 2									
6.	XAP-108	MH	NA	+ +	30.67	MR	26.7	3 weeks	low
7.	XAP-83	MH	NA	+ +	33.33	S	42.9	2 weeks	high
8.	XAP-110	TN	NA	- +	30.33	MR	NA	NA	NA
9.	XAP-134	MH	No	+ +	26.30	R	45.3	3 weeks	high
10.	XAP-104	MH	R-H	+ +	27.41	R	34.2	3 weeks	high
11.	XAP-116	RJ	No	- -	37.04	S	43.8	2 weeks	high
12.	XAP-97	KA	R-H	- +	33.33	S	59.7	3 weeks	high
13.	XAP-96	MH	N-T, S-F, A-D, Q-H, R-H	- +	32.96	S	38.4	2 weeks	high
14.	XAP-115	UP	No	+ +	30.43	MR	29.8	3 weeks	low
15.	XAP-114	MH	No	- +	33.33	S	62.3	3 weeks	high
16.	XAP-94	TL	No	- -	34.07	S	41.7	3 weeks	high
17.	XAP-99	MH	No	+ +	28.52	R	58.0	2 weeks	high
18.	XAP-92	HP	R-H	+ -	37.80	S	86.7	2 weeks	Very high
19.	XAP-98	MH	R-H	+ +	30.74	MR	51.4	2 weeks	high
20.	XAP-61	MH	R-H	+ +	27.78	R	48.1	3 weeks	high
SSR data not available									
21.	XAP-63	MH	Y-I (insertion)	+ +	26.67	R	NA	NA	NA
22.	XAP-88	MH	No	+ +	27.52	R	NA	NA	NA

+ -: presence or absence of gene *strA/b*, R: resilience, S: sensitivity, and MR: moderate resilience.

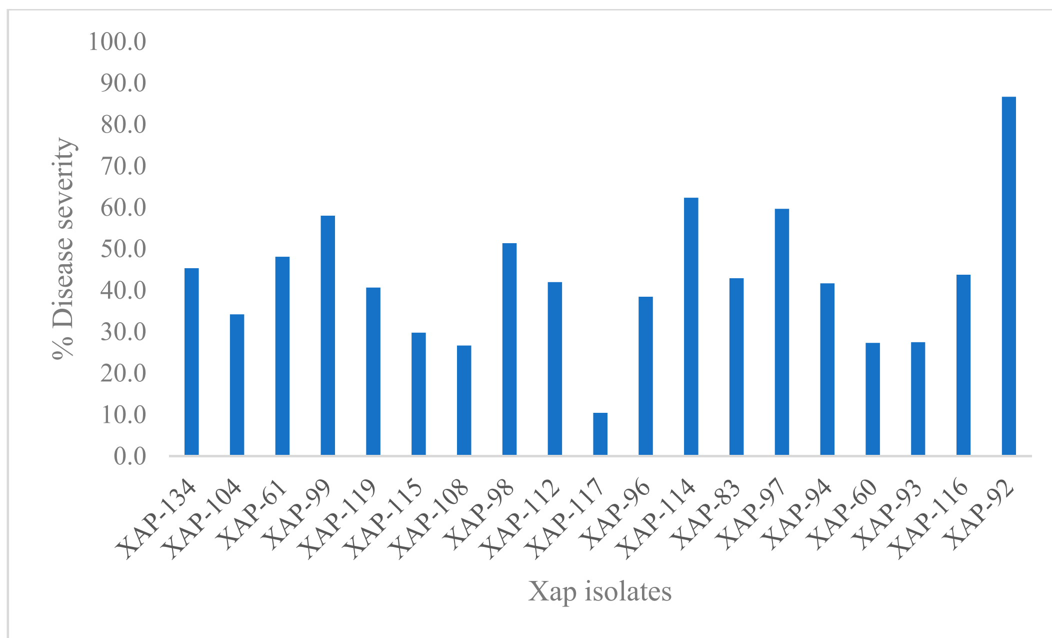

Figure 2. Percentage of disease severity (% DS) caused by different Xap isolates (n = 22) on healthy Bhagwa pomegranate plants under greenhouse conditions.

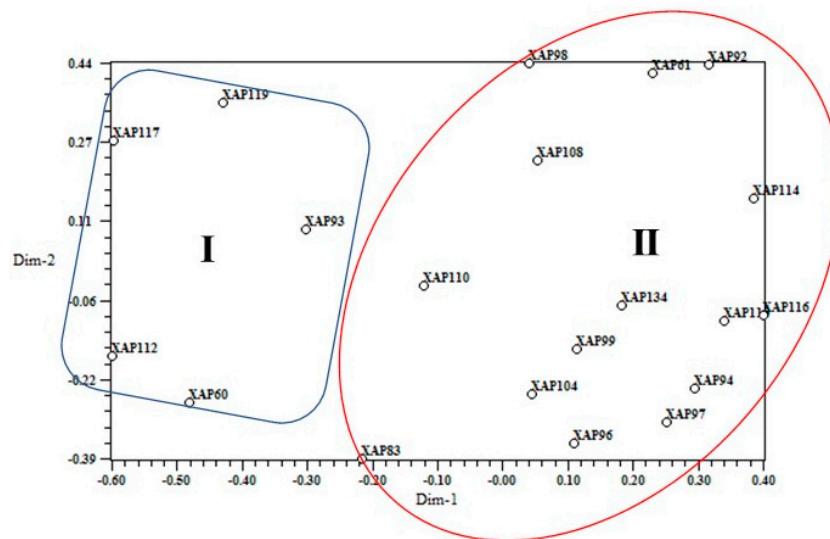

Figure 3. Dendrogram showing genetic diversity amongst Xap isolates on the basis of SSRs.

3.2. Sensitivity to Streptomycin and Its Molecular Basis

Sensitivity to streptomycin was checked for in all the isolates in vitro. These isolates could grow on media containing 250 µg/ml to 1500 µg/ml streptomycin. The zone of inhibition at the highest concentration (1500 µg/mL) was below 50% (ranged between 26.3% and 37.8%), indicating a lower sensitivity to the antibiotic. Notably, seven isolates had less than 30% growth inhibition, indicating the lowest sensitivity in these isolates (Table 5). A positive correlation was found between the concentration of streptomycin and ZOI (Table 5), i.e., as the concentration increased, the ZOI also increased, indicating a higher sensitivity at a higher concentration of the antibiotic. However, no correlation could be deduced between % DS and ZOI (Figure 4). Nonetheless, an interesting observation was that the isolate (#92) with the highest % DS (86.7%) also had the highest ZOI (37.8), which means the most pathogenic isolate (current study) was also the most sensitive to the antibiotic (Figure 5). To analyze the molecular basis of the observed lower sensitivity to the antibiotic, the presence of the *strAB* gene pair was confirmed through PCR amplification using gene-specific primers (Figure S3a,b). A total of 90% of the isolates showed the presence of either part of the gene pair *strA-strB,* of which 60% of the isolates showed the presence of the complete gene pair *strAB* (Figure S3a,b). In addition, the *rpsL* gene was amplified in all the isolates using gene-specific primers, and the amplicons were sequenced. The nucleotide sequence alignment revealed G to A transitions in six of the isolates (Figure 5), resulting in a change in the amino acid sequence (Figure 6). Clustering based on the *rpsL* amino acid sequence revealed distinct clusters (Figure 7).

Table 5. In vitro sensitivity of Xap isolates to different concentrations of streptomycin.

Isolates Treatment (µg/mL)	XAP-61	XAP-63	XAP-88	XAP-99	XAP-104	XAP-114	XAP-119	XAP-134	XAP-93	XAP-117	XAP-96
250	22.326 (14.44)	24.074 (16.67)	22.622 (14.81)	20.111 (11.85)	23.504 (15.93)	26.177 (19.47)	26.296 (19.63)	22.622 (14.81)	24.651 (17.41)	27.597 (21.48)	28.114 (22.22)
500	23.509 (15.93)	27.151 (20.84)	25.805 (18.96)	20.111 (11.85)	23.509 (15.93)	30.364 (25.56)	27.603 (21.48)	26.296 (19.63)	27.85 (21.85)	32.515 (28.89)	31.32 (27.04)
750	27.861 (21.85)	30.351 (25.56)	29.868 (24.81)	27.34 (21.11)	26.818 (20.37)	33.664 (30.74)	29.377 (24.07)	28.114 (22.22)	29.61 (24.44)	34.817 (32.59)	36.151 (34.81)
1000	30.118 (25.19)	29.627 (24.44)	30.845 (26.30)	28.377 (22.59)	27.34 (21.11)	32.979 (29.63)	30.838 (26.30)	28.627 (22.96)	34.128 (31.48)	34.809 (32.59)	35.935 (34.44)
1250	31.071 (26.67)	31.562 (27.41)	31.562 (27.41)	30.351 (25.56)	27.58 (21.48)	33.203 (30.00)	32.515 (28.89)	28.864 (23.33)	35.487 (33.70)	35.035 (32.96)	35.706 (34.07)
1500	31.806 (27.78)	31.071 (26.67)	31.635 (27.52)	32.273 (28.52)	31.562 (27.41)	35.255 (33.33)	32.274 (28.52)	30.849 (26.30)	35.939 (34.44)	35.035 (32.96)	35.035 (32.96)
Control	0.309 (00.0)	0.309 (00.0)	0.309 (00.0)	0.309 (00.0)	0.309 (00.0)	0.309 (00.0)	0.309 (00.0)	0.309 (00.0)	0.309 (00.0)	0.309 (00.0)	0.309 (00.0)
S.Em (±)	0.59	0.67	0.51	0.52	0.66	0.55	0.57	0.57	0.62	0.50	0.43
CD at 0.01	2.468	2.823	2.159	2.177	2.787	2.31	2.400	2.405	2.601	2.106	1.836
CV (%)	4.258	4.664	3.590	3.946	4.984	3.474	3.863	4.172	3.987	3.039	2.606
Correlation	0.96	0.93	0.92	0.96	0.94	0.88	0.98	0.93	0.97	0.81	0.78
p-value	0.001184	0.00358925	0.004672	0.001184	0.002646	0.010368	0.000298	0.00358925	0.00066825	0.02536025	0.033638
250	28.871 (23.33)	27.334 (21.11)	29.868 (24.83)	31.455 (27.27)	29.121 (23.70)	28.873 (23.33)	25.208 (18.15)	27.603 (21.48)	27.861 (21.85)	26.017 (19.26)	29.121 (23.70)
500	30.845 (26.30)	28.117 (22.22)	31.321 (27.04)	27.861 (21.85)	30.605 (25.93)	31.562 (27.41)	29.121 (23.70	30.605 (25.93)	29.377 (24.07)	28.114 (22.22)	32.741 (29.26)
750	32.274 (28.52)	29.357 (24.07)	34.359 (31.85)	36.825 (35.93)	35.248 (33.34)	34.807 (32.59)	28.868 (23.33)	34.807 (32.59)	29.86 (24.81)	28.627 (22.96)	35.264 (33.33)
1000	32.747 (29.26)	31.564 (27.41)	34.809 (32.59)	35.699 (34.07)	33.671 (30.74)	34.809 (32.59)	29.377 (24.07)	34.809 (32.59)	32.038 (28.15)	29.868 (24.81)	36.374 (35.19)
1250	33.669 (30.74)	32.274 (28.52)	35.258 (33.33)	37.041 (36.30)	33.669 (30.74)	35.036 (32.96)	30.364 (25.56)	35.264 (33.33)	32.038 (28.15)	31.088 (26.67)	36.826 (35.93)
1500	33.661 (30.74)	33.62 (30.67)	35.255 (33.33)	37.933 (37.80)	35.706 (34.07)	35.255 (33.33)	33.932 (31.19)	35.704 (34.07)	33.415 (30.33)	33.475 (30.43)	37.485 (37.04)
Control	0.309 (00.0)	0.309 (00.0)	0.309 (00.0)	0.309 (00.0)	0.309 (00.0)	0.309 (00.0)	0.309 (00.0)	0.309 (00.0)	0.309 (00.0)	0.309 (00.0)	0.309 (00.0)
S.Em (±)	0.50	0.69	0.66	0.67	0.70	0.67	0.69	0.67	0.56	0.58	0.51
CD at 0.01	2.108	2.907	2.783	2.814	2.948	2.807	2.906	2.813	2.342	2.420	2.152
CV (%)	3.153	4.589	3.988	3.910	4.285	4.023	4.710	4.064	3.658	3.942	2.989
Correlation	0.95	0.99	0.91	0.79	0.83	0.87	0.91	0.89	0.98	0.98	0.93
p-value	0.0018	7 E-05	0.0059	0.0308	0.0204	0.0121	0.0059	0.0087	0.0003	0.0003	0.0036

Note: values represent treatment means of arc sin transformed data points. Values in the parentheses are treatment means of non-transformed data points.

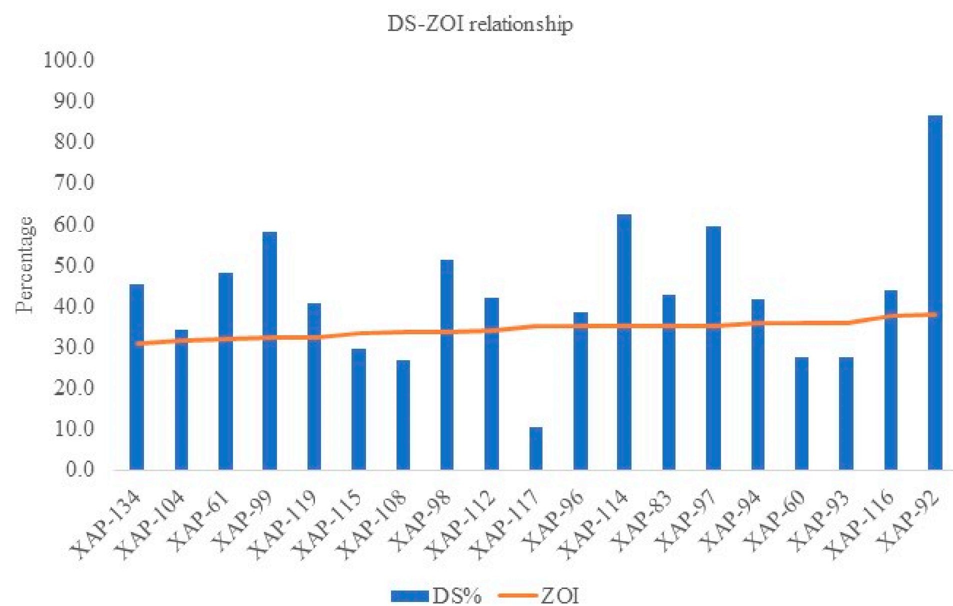

Figure 4. Relationship between percent disease severity (% DS) observed after 6 weeks and zone of inhibition (ZOI) at the highest concentration (1500 µg/mL).

Figure 5. Multiple sequence alignment of *rpsL* nucleotide fragment amplified from Xap isolates and the reference sequence retrieved from NCBI database.

Figure 6. Multiple sequence alignment of rpsL protein from Xap isolates and the reference sequence retrieved from NCBI database using ClustalW.

Figure 7. Neighbor-joining tree on the basis of amino acid sequences using Max Lil.

4. Discussion

Xanthomonas axonopodis pv. *punicae* (Xap), which causes bacterial blight in pomegranates, can lead up to 100% yield loss to the grower. Blight-infected fruits also have a low marketability. The pathogen was first reported from India in 1952 from Delhi [28]. Over the years, the disease has been reported from almost all pomegranate-growing regions of India [29–32], as well as other countries, such as Pakistan [33], South Africa [34], and Turkey [35]. The isolates obtained in the current study originated from different geographical locations spanning north India (HP, UP) to the southern-most state of India (TN). This study also included the maximum number of isolates from Maharashtra, which is the leading producer of pomegranates in India and an endemic area for the bacterial blight disease (Supplementary Figure S1), to provide a comprehensive view of the spread of the pathogen. These isolates resembled Xap morphologically and in their growth characteristics, for example, yellow-colored colonies producing the characteristic brown pigment, fuscan [21,22].

To analyze their pathogenic potential, the spray inoculation method was used in the current study, which has been reported as an efficient inoculation technique, causing symptoms to appear within 21 days under optimal conditions (temperature and humidity) and leading up to 67.8% disease severity [21]. In accordance with our previous results [21], all the isolates showed characteristic blight symptoms on healthy Bhagwa leaves 14–21 days post inoculation and disease severity up to 86%. Leaf lesion areas were also recorded; however, no correlation was observed between disease severity and lesion area (data not shown). The ability of the isolates to produce characteristic blight symptoms is an indication that these bacteria are Xap, since the close relatives of Xap, such as *X. campestris* pv *campestris* and *X. axonopodis* subsp. *citri*, fail to infect pomegranates [23]. Apart from identification based on phenotypic traits, early and reliable molecular detection of Xap even before the symptoms are manifested on the host plant can aid in devising suitable control measures for the disease and ensure good quality and quantity of fruits harvested. Molecular identification can be performed based on the amplification of the *gyrB* [36] and *XopQ* [23] genes. The LAMP (loop-mediated isothermal amplification) technique has also been employed for early detection of Xap infection on pomegranate plants [37]. The identity of Xap isolates in the current study was confirmed at the molecular level based on the PCR amplification of XopQ effectors (Figure 1), [23]. These XOPQ effectors in the *Xanthomonas* species belong to the type III secretion system, which interferes with the plant immune response [38], and therefore are integral to *Xanthomonas*–host interaction.

Next, to assess genetic diversity amongst the isolates, eighteen SSR markers were used. SSRs are hypermutable and highly polymorphic, and therefore useful in the analysis of genetic relationships amongst prokaryotic organisms [39]. In prokaryotic organisms, SSRs have been found to be located in promoter regions or reading frames of a set of genes (contingency loci) which are involved in pathogen–plant interaction [40,41]. SSRs have also been found to be associated with virulence factors [42] and were able to reveal pathogenic variability in bacterial pathogens such as *Xanthomonas albilineans*, causing leaf scald in sugarcane [43], as well as fungal pathogens such as *Alternaria carthami*, causing leaf spots in safflower [44]. In the current study, SSRs proved useful in assessing the genetic diversity of Xap isolates, which could be divided into two clusters. The isolates with extremely different levels of pathogenicity were grouped into separate clusters (Figure 3), indicating that the SSRs used in the current study could also reveal pathogenic variability amongst Xap isolates. For example, isolate number Xap 92 was found to be genetically diverse from other isolates, and this genetic diversity probably contributed to its high pathogenicity. This isolate belonged to ST3, which contained isolates obtained from various geographical locations in India [22]. Four sequence types (ST1–4) were obtained when multi-locus sequence analysis (MLSA) was performed on Xap isolates obtained from different geographical locations, indicating the clonal nature of the pathogen. This was attributed to the monoculture of the susceptible cultivars of pomegranates with high genetic homogeneity [22]. Moreover, the markers could not cluster the isolates based on their geographical locations, indicating the high adaptability of the pathogen to diverse climatic conditions. This could be a cause

for concern, because if highly pathogenic isolates become prevalent or evolve into even more pathogenic isolates, then it would certainly hamper pomegranate quality, affecting its export and other pomegranate-based industries throughout the country.

To check the incidence and growth of *Xanthomonas*, pomegranate growers often use streptocycline in the field. Streptocycline is a mixture of streptomycin and tetracycline in a ratio of 9:1. However, in recent years, lower sensitivity (higher resistance) to streptomycin has been reported by farmers. Streptomycin sensitivity is controlled by genes present on plasmid/transposon-borne or chromosomes [9]. Plasmid-borne resistance is mainly due to the presence (and activity) of *strA* and *strB* (*strAB*) genes. Chromosomal mutation is, however, due to mutations in the *rpsL* gene that encodes for a ribosomal protein subfamily. In *Erwinia amylovora*, the causative agent of fire blight in the *Rosaceae* family, resistance due to *strAB* gene activity, confers a high level of insensitivity (MIC values 500–750 μg/mL), while resistance due to chromosomal mutation confers a low level of insensitivity (MIC values 2000 μg/L) [45]. Resistant strains of *Xanthomonas citri* subsp. *citri* (causative agents of citrus canker) efficiently grew on media supplemented with 100 μg/mL streptomycin, while growth of susceptible strains was inhibited at this concentration [18]. Therefore, in the current study, we used a range of antibiotic concentrations (250 μg/ml to 1500 μg/mL) to check the level of antibiotic sensitivity in the Xap isolates, which was measured in terms of ZOI (Table 5). Lower ZOI indicates lower sensitivity to the antibiotic. All the isolates could grow on media containing 1500 μg/ml of streptomycin, indicating a higher resistance of the isolates. Moreover, the lowest ZOI at 1500 μg/mL observed in the current study (26.3%) was lower than that reported in other studies (33.33%) [20], indicating a higher resistance level of the Xap isolates in the current study. The probable reason for this difference could be that the earlier study [20] included isolates only from Karnataka; however, our study included isolates from other geographical regions, as well. Consistently, all the isolates showing lower sensitivity to the highest concentration of the antibiotic (ZOI < 30%) in vitro were isolated from pomegranate orchards in Maharashtra. Since Maharashtra is the leading pomegranate-producing state in India, it can be expected that the exposure to the antibiotic spray in the field would also be high in this state. Therefore, the ecological reason for the acquired resistance could be the greater application of the antibiotic in pomegranate-growing regions of Maharashtra, hence greater exposure of the pathogen to the antibiotic and the resultant high selection pressure. Interestingly, the isolate with highest ZOI was obtained from pomegranate orchards in Himachal Pradesh, which is a not-so-dominant region in pomegranate cultivation, hence lower exposure of Xap to the antibiotic spray and less selection pressure.

The molecular basis of the acquired resistance could be horizontal gene transfer, as highlighted by the observations in the current study that all the isolates showing less than 30% growth inhibition (at 1500 μg/mL antibiotic concentration) showed amplification of the gene pair *strAB*. On the other hand, only *strA* could be detected in the isolate exhibiting the highest sensitivity to the antibiotic, indicating the absence of the complete gene pair (*strAB*) that is required for antibiotic resistance. Such transposon-mediated resistance to streptomycin due to the horizontal transfer of *strAB* genes has also been reported in other species of *Xanthomonas*, such as *X. campestris* or *X. smithii* or different pathovars *Xanthomonas axonopodis* pv. *vesicatoria* [46,47].

Mutation in the *rpsL* gene leading to alteration in the antibiotic binding site of ribosomes is also an important mode of acquired antibiotic resistance. Transitions (G to A) were observed in some of the isolates investigated; however, there were no changes in the conserved regions. Many studies reported a conserved mutation, lysine to arginine at 43rd position (K43R), in the *rpsL* gene that is responsible for antibiotic resistance in clinical as well as plant pathogens [48–50]. However, we did not find the occurrence of this mutation in the isolates used in the current study. Moreover, there are several other mutations reported in *Erwinia amylovora* that may have a role in the improved fitness of the pathogen under the selection pressure imposed by the presence of the antibiotic [50]. Therefore, the mutations observed in the current study probably have a role in the improved

growth of Xap on antibiotic-containing media. Nevertheless, to ascertain their specific role in antibiotic resistance, more elaborate future studies employing Xap mutants would be needed.

5. Conclusions

In the current study, isolates of *Xanthomonas axonopodis* pv. *punicae* (Xap), a major bacterial pathogen of the pomegranate, were obtained from orchards covering different geographical locations in India. The results of the study highlight the variability in the pathogenic potential of these isolates, with the disease severity on the host plants ranging from 10% to 86%. Further, the isolates exhibited genetic diversity, as assessed by SSR markers, which could divide the isolates into two clusters, with the isolates showing the highest pathogenic variability falling into separate clusters. These markers could not cluster the isolates based on their geographical locations, indicating the high adaptability of the pathogen to diverse climatic conditions. The development of antibiotic resistance in pathogens is a serious cause for concern. The problem gets aggravated when the resistance is exhibited by an economically important pathogen such as Xap. Use of streptocycline, containing streptomycin sulphate and tetracycline sulphate (9:1), had been effective in checking the growth of Xap in pomegranate orchards. However, in recent years, there has been a decline in its effectivity, which could be attributed to the acquired resistance by the bacteria. Based on the results of the current study, we conclude that the isolates of Xap prevalent in the major pomegranate-growing regions of India have acquired resistance to streptomycin and that the level of this resilience is higher than other *Xanthomonas* species. This acquired resistance could be due to the horizontal transfer of *strA/B* genes or due to mutations in the *rpsL* gene, as observed in the study. Moreover, if the mutations observed in these isolates are involved in the improved fitness of the pathogen under the influence of antibiotics, then it could have serious ecological repercussions. In view of the emerging antibiotic resistance in plant pathogenic bacteria, the latest guidelines that prohibit the use of antibiotics in India is a timely and welcome decision. However, post the ban on the use of antibiotics, there is an urgent need to develop high-fidelity alternate strategies to manage and control this destructive pathogen of the pomegranate, an economically important cash crop in India.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/horticulturae8050441/s1, Figure S1: Geographical locations in India from where Xap was isolated. Figure S2: Jaccard's similarity coefficients for different Xap isolates. Figure S3: Gel electrophoresis image showing the amplification of (a) *strA* and (b) *strB* genes involved in streptomycin metabolism.

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